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THE ENZYMES OF PYTHIACYSTIS CITROPHTHORA Sm. AND Sm.[1-2]

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I. INTRODUCTION

This paper reports a study of the enzymes produced by the fungus, *Pythiacystis citrophthora*, when grown in pure culture. For references and short general reviews of the several works on the enzymes of the various groups of fungi, see Waksman,(25) Waksman and Davison,(26) Dox,(6) Zeller(27) and Cooley.(5) Considering forms morphologically rather near to Pythiacystis, Emoto(7) found in species of Saprolegnia that amylase, inulase, raffinase, invertase, lactase, maltase, emulsin, salicase, glycolase, proteolytic enzymes (acid, neutral, alkaline), peroxidase and catalase were present as intra-cellular enzymes, and pectinase, cellulase, lipase, urease, tyrosinase and oxidase were absent. In the Achlya, amylase, inulase, cellulase, invertase, lactase, maltase, emulsin, salicase, proteolytic enzymes (acid, neutral, alkaline) peroxidase, and catalase were present and pectinase, glycolase, lipase, urease, tyrosinase and oxidase absent.

Among the enzymes reported by various workers as being produced by representatives of the Mucorales are cytase, cellobiase, pectinase, inulase, diastase, maltase, invertase, lactase, zymase, rennet and lipase. This briefly sums up the known work on the enzymes produced by the phycomycetous fungi.

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[1] Paper No. 161, University of California Graduate School of Tropical Agriculture and Citrus Experiment Station, Riverside, California.

[2] This is the first of a series of papers to appear on the parasitism of *Pythiacystis citrophthora*. The work is being carried on in the laboratory of and under the direction of H. S. Fawcett to whom the writer is indebted for many helpful suggestions.

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II. MATERIAL AND METHODS

The organism used in the following work was a virulent strain of *Pythiacystis citrophthora* isolated by H. S. Fawcett of the Citrus Experiment Station and by him given the number 1309a. The pathogenicity of the fungus has been studied by Smith and Smith[21] in connection with the fruit rot of citrus and by Fawcett[8, 9] in connection with the gummosis disease. The virulence of this particular isolation was tested by hundreds of inoculations into the fruit and bark of various citrus species. It was grown for 10 days at room temperature (18–25° C) on glucose-potato-peptone broth made of the following materials:

1. Watery extract from 250 gm. of peeled, sliced and boiled potatoes
2. Dextrose, 20 gm.
3. Bacto-Peptone, 10 gm.
4. Water to make 1000 ml.

The mats were filtered on a Buchner funnel, washed with distilled water, and divided into two equal lots. One lot was desiccated and made permeable by treatment with acetone and ethyl ether according to the acetondauerhefe method of Albert, Buchner and Rapp.[1] See also Kohnstamm,[14] Onslow[16] (p. 23) and Dox[6] (p. 38). The coarse powder resulting was ground to a fine flour and stored in brown glass bottles. The other lot was not treated with acetone and ether but was dried in an electric oven at 45° C and then ground to a fine flour and added to the acetondauerhefe preparation, assuring the presence of enzymes that may have been destroyed by the first method. This material was the source of the enzymes throughout the work.

In the first experiment an extract was made of the fungus powder by grinding it with glass in twenty times its weight of distilled water. The suspension was allowed to extract for an hour, then filtered through filter paper, and finally through the finest Mandler filter candle. The last operation was done under aseptic conditions so that a sterile enzyme extract was obtained. This was added aseptically in 5-ml. quantities to 20 ml. of 1 per cent solutions of the various substrata, making use of a modification of the apparatus described by Smith.[21] In this series, therefore, it was unnecessary to add toluene, chloroform or other preservative. The enzyme "cultures" were incubated in the dark at 25° C for 8 days. The extract thus prepared showed a slight urease and maltase reaction, but no protease, no deaminase or deamidase with asparagin, and no invertase. Evidently only a small quantity of the enzymic materials passed the filter candle.

In all the subsequent work, unless otherwise specified, 20 cc. of 1 per cent solutions or suspensions of the various test materials were added directly to 250 mgm. of the enzyme powder in 200 ml. Erlenmeyer flasks, the system preserved with 1 ml. of C. P. toluene, and the flasks tightly stoppered. The "cultures" were incubated in the dark at temperatures ranging from 29° C to 39° C. For every culture prepared in this way a parallel check was run using enzyme powder that had been autoclaved at 16 pounds pressure for 15 minutes.

As further precautions blank determinations were made on extracts of the active and autoclaved powders as follows: (*a*) reducing power in Fehling's solution, (*b*) amino nitrogen (*c*) ammonia nitrogen, (*d*) total acidity and (*e*) active acidity. The various works on fungus enzymes have in general ignored the probability of an important difference between the powder before and after autoclaving. To determine the reducing power the iodometric method of Shaffer and Hartmann[20] was employed. Amino nitrogen was estimated by the van Slyke[24] micro method, ammonia nitrogen by Folin's aeration method [see Shaffer ('03)], and active acidity by the colorimetric method, using the indicators and buffers recommended by Clark.[4] Total acidity was determined by direct titration against standard alkali, phenolphthalein being used as an indicator. The reducing power is shown by the following determinations:

TABLE 1

REDUCTION OF FEHLING'S SOLUTION BY FUNGUS POWDER

Material	Incubation period (*days*)	Temperature (°*C.*)	Reducing power (mgm. Cu liberated by 1 ml. of extract)	
			Active	Autoclaved
.25 gm. enzyme powder+20 cc. H_2O +1 cc. toluol.	3	37	4.95	.6192
	4	39	3.16	.894
	10	38	6.88	.743
	21	38	4.599	.8256
	1½	40	2.614	.8944
	1½	40	2.924	.9976
	1½	40	3.37	.86

The inconsistencies in the above determinations are accounted for by the small reducing power of the extracts, which is below the minimum of 10 mgm. per ml. (or 1 mgm. per ml. when diluted to 50 ml.) recommended for accuracy by the authors. Small as these values are they are important because they negate what otherwise

would seem to be evidence for the presence of some specific carbohydrases in the enzyme powder.

Moreover, it is seen that autoclaving brings about a marked loss in the ability of the powder to reduce Fehling's solution. This is probably due in part to the destruction of the aldehyde groups of the dextrose absorbed by the mycelium; and is effected by the union of the aldehyde groups with the indole groups of the fungus proteins resulting in the formation of the dark-colored humin nitrogen. [See Gortner and Holm.[10]] The fungus powder was changed from a light gray to a dark brown by the autoclaving which may indicate that such a transformation had taken place.

TABLE 2

TOTAL AND ACTIVE ACIDITY, AND AMINO AND AMMONIA NITROGEN OF THE ENZYME EXTRACTS

Incubation period	Temp.	Total acidity (ml. .098 N NaOH to neutralize 10 ml. of extract to phenolphthalein)		Active acidity (P_H)		Amino N (N_2 from 2 ml. ext.) ml.		Ammonia N (ml. .0952 N H_2SO_4 to neut. 5 cc. ext. to phenolphthalein)	
(*days*)	(°*C.*)	Active	Autoclaved	Active	Autoclaved	Active	Autoclaved	Active	Autoclaved
10	38	.45	.45	5.8	4.8	.44	.278	.35	.35

Taking these considerations into account it is evident that the autoclaved powder does not serve as a true check. Accordingly in the calculations, the reducing power of the active powder alone plus the reducing power of the substrate to which the autoclaved, inactivated powder had been added is substracted from the reducing power of the system with the active enzyme powder, and to this result is added the reducing power of the autoclaved enzyme. This reasoning is necessarily based upon the assumption that the catalytic effect of the fungus powder, other than its enzymic action, is similar in both the active and autoclaved powder. An example will illustrate the procedure.

Suppose

		Mgm. Cu.
	(1) Starch solution plus active enzyme gave a reduction	25
and	(2) Active enzyme alone	5
and	(3) Starch solution plus autoclaved enzyme	2
and	(4) Autoclaved enzyme alone	1

The reduction due to the hydrolyzed starch is evidently not 25 minus 2 equal 23, but 25 minus 5 minus (2 minus 1) equals *19* if we make the assumption stated above. That assumption was made in the calculations of this work.

ESTERASES OR LIPASES

As test substrata for the determination of esterases, olive-oil emulsion, lemon oil emulsion, cream, methyl acetate, and ethyl acetate were used. The degree of change in active and total acidity was taken as the criterion for the presence or absence of these enzymes. A slight modification of the method of Rice and Markley[19] was used to test for lipase where cream was the substrate. The cream was heated to destroy any lipase present and then saturated with cane sugar as a preservative. The enzyme powder was added and the cultures incubated. The

TABLE 3

ACTION OF ESTERASES OF *Pythiacystis Citrophthora* ON VARIOUS SUBSTRATES

Substrate	Incubation period (*days*)	Temp. (°C.)	Total acidity (Vol. .098N NaOH to neut. 10 ml. substrate)		Active acidity (PH)	
			Active	Autoclaved	Active	Autoclaved
Olive oil	4	38	.30	.30	5.8	5.4
Lemon oil	4	38	1.00	1.05		
Cream	4	38	2.35	.85		
Methyl acetate	4	38	1.15	0.40	4.8	4.9
Ethyl acetate	4	38	1.20	0.45	4.5	4.9

acidity was determined at the beginning and at the end of incubation by diluting an aliquot with ten times its volume of water, and titrating to a phenolphthalein endpoint. The advantages of the method are pointed out by the authors. Cream is a well emulsified, natural fat, and the sugar preservative increases the viscosity and keeps the fat from separating for a long time. A disadvantage is an indistinct endpoint in the titration, but that is less troublesome if the aliquot is diluted. The principle of Bloor's[3] method, as described by Zeller[27] was used in preparing the olive oil emulsion. In this method 1 ml. of olive oil in 10 ml. of hot absolute alcohol was drawn into 100 ml. of cold distilled water by means of a suction flask fitted with a funnel having a capillary delivery end. The alcohol was then boiled off and the enzyme powder added. With the lemon oil no alcohol was used, the oil being sucked directly into the watery suspension of the enzyme powder. As usual toluol was used as a preservative.

The results show that the enzyme powder is capable of increasing the acidity of some of the ester substrates. This must be interpreted as being due to the production of fatty acids by hydrolysis. It may be

said here that none of these esters and oils used in 1 per cent strength in synthetic media having no other carbon source supported growth of the fungus. Cream was not tried in that way. Autoclaved cream alone did support growth. Lemon oil added to autoclaved rind and albedo was distinctly inhibitive to the growth of the organism.

CARBOHYDRASES

Table 4 shows the test materials used to demonstrate the presence or absence of the respective carbohydrases. The data for reducing power represent the values after the checks have been substracted according to the manner already described.

TABLE 4

ACTION OF FUNGUS POWDER ON VARIOUS CARBOHYDRATES

Substrate	Incubation period (*days*)	Temp. (°C.)	Reducing power (mgm. Cu liberated from Fehling's* sol. by 1 ml. of substrate)
1. Lintner's soluble starch suspended in cold H_2O	4	39	2.335
2. Lintner's soluble starch suspension autoclaved	3	37	17.060
3. Lintner's soluble starch, autoclaved	1½	40	13.8976
4. Potato starch, cold H_2O	4	39	2.200
5. Potato starch suspension, autoclaved	4	39	16.579
6. Potato starch suspension, autoclaved	2	29	12.454
7. Inulin, cold H_2O	4	37½	.0402
8. Inulin, cold H_2O autoclaved	3	37	.9612
9. Inulin, cold H_2O	22	38	2.2684
10. Hemicellulose	12	25	2.578
11. Hemicellulose	23	25	3.9196
12. Hemicellulose	22	38	0.136
13. Cellulose	4	37½	0.00
14. Cellulose	16	38	0.00
15. Cellulose	22	38	0.00
16. Raffinose	3	37	4.4014
17. Lactose	2	29	2.6812
18. Lactose	3½	39	2.062
19. Maltose	2	29	8.667
20. Sucrose	2	29	40.2148
21. Sucrose	4	37	21.46*
22. Sucrose	1½	40	15.7208*
23. Sucrose	1½	40	4.9332†

* Different lot of enzyme powder used.

† Different lot of enzyme powder and a 10 per cent sucrose solution used.

Of the polysaccharides used (starch, inulin, hemicellulose, and cellulose) the first was the only one strongly hydrolyzed by the fungus powder, and this only after the starch had been gelatinized by heating in water. The Lintner's soluble starch and the potato starch behaved similarly. The presence of diastase is established. Inulin showed a slight reduction only after a long incubation with the powder. This may be partly explained by the low incubation temperature and low hydrogen ion concentration used. Pringsheim and Kohn[17] found the optimum temperature for the action of this enzym to be 55° C, and the optimum hydrion concentration to be P_H 3.8. No attempt was made to induce inulase activity in the organism by growing it in the presence of the carbohydrate. This phase was not considered with any of the test materials employed in this work. However, it is observed that peptone and starch were present in the nutrient in which the organism was grown. The peptone, as will be seen later, did not induce protease formation.

Hemicellulose was slightly hydrolyzed as is evidenced by the small reduction of Fehling's solution, and this may be taken to indicate cytases. The hemicellulose was prepared from date endosperm by the method of Zeller.[27] There was no cellulase activity exhibited. The test material in this case was filter paper cellulose prepared by the method of McBeth and Scales.[15] Further attention to the cyto-hydrolyzing enzymes is being given in connection with some histological and microchemical work now in progress.

Pectinase was tested for by comparing the ability of active and autoclaved powder to macerate living tissues. Disks of potato tuber, 400 mm. in thickness, were made by use of a cork borer and sliding microtome. The disks were placed in water extracts of the enzyme powder and the "cultures" preserved with toluol. At intervals disks were removed and their coherence tested. Scarcely any difference could be detected up to the 16th day of incubation, when the disks in the active extract tore slightly more easily than those in the autoclaved extract. Carrot and red beet disks were similarly tested; there was no apparent difference between the active enzyme extract and the check. The amount of red pigment of the beets that diffused into the extract of active enzyme was not greater than that with the autoclaved powder. The enzyme powder would not coagulate commercial pectin ("Certo").

Of the simple carbohydrates, raffinose and maltose were appreciably hydrolyzed. Sucrose was very strongly inverted. Of the three disaccharides lactose was least attacked.

In order to test the ability of the fungus to use these carbohydrates as sources of carbon they were added in quantities making 1 per cent of the total volume to synthetic media of the following formulae:

	Grams per liter		
$MgSO_4 \cdot 7\ H_2O$	0.50	0.50	.50
K_2HPO_4	1.00	1.00	1.00
KCl	.50	0.50	.50
$NaNO_3$	2.00	2.00 $Ca(NO_3)_2$	10.00
$FeSO_4$	.01	.01	.01
Agar	20.00	0.00	20.00

A comparison of the amount of extension of the mycelium in the carbohydrate cultures with that in the checks having no carbon other than that as agar, CO_2, impurities, and that stored in the mycelium indicated that all the carbohydrates could be used by the fungus, with the possible exceptions of lactose, inulin, and cellulose. The colonies in these media showed only slight increase in growth over those of the checks, and the cellulose medium showed no clearing zone near the fungus. It must be said, however, that such observations were unsatisfactory, owing to the poor growth of the organism on these synthetic media.

GLUCOSIDASES

Five glucosides were used to test for the respective glucosidases as shown in the following table. Here again the results represent net reduction after deduction for the checks:

TABLE 5

ACTION OF THE FUNGUS POWDER ON GLUCOSIDES

Substrate	Incubation period (*days*)	Temperature (°*C.*)	Reducing power (mgm. Cu liberated from Fehling's sol. by 1 ml. of substrate)
1. Hesperidin	2	29	1.8556
2. Phloridzin	3	37	5.285
3. Amygdalin	3	37	13.895
4. Salicin	3	37	16.6476
5. Arbutin	2	29	49.7404

The strikingly large hydrolysis of amygdalin, salicin and arbutin prove the presence of the B-glucosidase (emulsin) in the Pythiacystis. In the case of amygdalin a strong odor of benzaldehyde was soon very evident in the enzyme cultures and also in the fungus cultures having amygdalin as a source of carbon. A solution of arbutin in the presence of the enzyme powder or inoculum of the fungus soon acquired the

color of a quinole solution. Although, as stated by van Rijn,[23] phloridzin is not attacked by emulsin, there is evidence here that the Pythiacystis can slowly bring about its hydrolysis. Judging by the small amount of reduction obtained, the presence of a glucosidase capable of hydrolyzing hesperidin is doubtful. One per cent phloridzin and arbutin as carbon sources were incapable of supporting growth of Pythiacystis on synthetic agar media; salicin and amygdalin permitted a small extension of the mycelium; and hesperidin still a smaller amount. It should be reiterated, however, that the organism makes such a poor growth on synthetic media in general that these comparative observations are not satisfactory.

Although the fungus was capable of tolerating a concentration of 0.1 per cent tannin (Merck's digallic acid) in glucose-potato-peptone broth medium, and .05 per cent in prune broth and Czapek's synthetic medium, no evidence has yet been secured for the presence of tannase. The official Procter-Löwenthal method [see Official Methods[2] ('21), p. 274] was used to determine tannin, and Jean's[13] [see Zeller,[27] p. 507] iodometric method for gallic acid.

AMIDASES

The three amino acids, alanine, tyrosine and asparagin were used to test for deaminases. The asparagin, being also an acid amide, may be used to reveal the presence of deamidases. Acetamide and urea were the other substances employed, the former for deamidase and the latter for urease. The aeration method of Folin was used to estimate the ammonia formed.

TABLE 6

Action of Fungus Powder on Amino Acids and Acid Amides

Substrate	Incubation period (*days*)	Temp. (°C.)	Initial (PH)	Final (PH)	Mgm. Ammonia N in 1 ml. of substrate. (Increase over check)
Alanine	4	37½			0.0
Alanine	23	37½		7.4	0.0
Tyrosine	4	37½			0.0
Acetamide	4	37		5.3	0.0
Acetamide	4	37		2.8	0.0323
Acetamide	4	37		5.2	0.0
Asparagin	2	29			0.0
Asparagin	5	37	9.0		1.067
Asparagin	5	37	2.4		0.0
Asparagin	23	37	8.6	8.7	.744
Urea	2	29			.865

Under the conditions employed no deaminase could be demonstrated and no deamidase with the acetamide. Asparagin gave a strongly positive test if the reaction was first adjusted to the alkaline side. Urease was demonstrated. The enzyme histozyme, which splits hippuric acid into glycine and benzoic acid, was tested for by determining the amino acid content of the substrate and by attempting to find benzoic acid after the incubation. The tests were negative, there being no increase in nitrogen liberated by nitrous acid, and no benzoic acid formed. In liquid media asparagin would serve as a source of N, and to a small extent as a source of both nitrogen and carbon. Urea would supply nitrogen, but not carbon and nitrogen in liquid media. The organism made no growth whatever on the agar media with urea present either with or without dextrose.

Acetamide appeared to be able to serve in a small measure as a source of nitrogen in liquid media, and very doubtfully as a source of both carbon and nitrogen. The tyrosine, alanine and hippuric acid were not tested in this way.

PROTEASES

In testing for proteoclastic enzymes an increase in aliphatic amino nitrogen, as determined in the van Slyke micro apparatus, was taken as positive evidence. Several other qualitative tests were employed, as will be explained.

TABLE 7

Action of Fungus Powder on Proteins

Substrate	Incubation period (*days*)	Temp. (°*C.*)	Initial (P$_H$)	Final (P$_H$)	N_2 gas (ml. from 2 ml. of substrate; increase over that of checks)
Peptone	2	29			0.00
Gelatine	2	29			0.00
Leucosine	6	37½	9.0	6.1	0.00
Leucosine	6	37½	7.0	5.9	.0695
Leucosine	6	37½	2.6	3.7	0.00
Leucosine	7	38			.3295

The leucosine was made according to the method described by Onslow[16] (p. 25). The qualitative tests for tryptophane with bromine water and amyl alcohol were negative. With the possible exception of the leucosine none of the materials were hydrolyzed. In

the two instances of the slight hydrolysis of leucosine the substrate was about neutral in reaction. Both the enzyme powder and the fungus itself failed to liquify nutrient gelatines, although the organism made a vigorous growth on this medium. The acetondauerhefe powder and inoculum of *Fusarium lycopersici* tried at the same time caused a rapid liquefaction of the gelatine.

In another experiment pulverized blood fibrin was stained with a one-half of one per cent aqueous solution of congo red, and then thoroughly washed and dried (see Reed[18]). One gram quantities of this were placed in 200-ml. Erlenmeyer flasks having the enzyme powder, 20 ml. of water, and 1 ml. of toluol. To some of the flasks were added 1 ml. quantities of .1 N H_2SO_4 or .1 N NaOH. The cultures were incubated 4 days at 37½ degrees C; this was followed by 12 days at room temperature (17–25 degrees C). In no case was there appreciable evidence of the protein having been attacked, which in this test is indicated by a liberation of the dye into the liquid. In the cultures having NaOH there developed a slight pink in the water showing that some Congo red had been liberated, but an amino-nitrogen determination did not reveal any increase in NH_2 groups. From these results one must conclude that proteoclastic enzymes are absent or at most very feeble in the enzyme powder employed. This material also failed to coagulate milk.

Qualitative tests were made for zymase, peroxidase, oxidase, catalase, reductase, and tyrosinase by the procedure described by Onslow[16] (pp. 23, 24, 25 and 128) and the presence only of peroxidase and catalase demonstrated.

A quantitative test was made for glycolase, the enzyme which decomposes dextrose to form lactic acid. To 20 ml. of an approximately 1 per cent glucose solution were added 250 mgm. of enzyme powder and 1 ml. of toluol; this system was incubated four days at 37° C. Loss in power to reduce Fehling's solution was taken as an indication of the presence of the enzyme. This loss amounted to 1.1008 mgm. Cu per ml. of solution and was assumed to indicate a weak glycolase activity. The solution with the active enzyme showed also a slightly higher total acidity than the one with the autoclaved enzyme. Waksman and Davison[26] (p. 249) point out that toluene is injurious to this enzyme, which fact may account for the small amount the glucose destroyed.

DISCUSSION AND SUMMARY

In a survey of the enzymes of the mycelium of *Pythiacystis citrophothora* the following were tested for: esterases, cellulase, cytase, pectinase (pectase), inulase, diastase, raffinase, invertase, lactase, maltase, emulsin (amygdalase, salicinase, arbutinase), glucosidases that attack hesperidin and phloridzin, tannase, amidases (deaminases, deamidases, urease) histozyme, proteases, rennet, zymase, peroxidase, oxidase, catalase, reductase, tyrosinase, and glycolase.

Very positive evidence was obtained for the presence of some of the lower esterases, for diastase, invertase, maltase, emulsin, phloridzinase, asparaginase, urease, peroxidase, and catalase; less evidence is forthcoming for the presence of cytase, lactase, hesperidinase; very slight indication of the presence of inulase, pectinase, protease and glycolase; and for the remaining enzymes sought the results were entirely negative.

The necessity for check determinations on both the active and deactivated enzyme material is emphasized, and a more accurate method of calculation given.

The diastase of this fungus attacks gelatinized starch vigorously, but starch suspended in cold water only feebly.

Although urea solution of the strength tried could not be used by the fungus, the enzyme powder gave a strong urease reaction.

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